Livestock Science 164 (2014) 57–66

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Estimation of methane emission using the CO_2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system

M.N. Haque *, C. Cornou, J. Madsen **

Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 2, DK-1870 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form
4 March 2014
Accepted 6 March 2014

Keywords:
Breath
CO_2-method
Methane
Production
Total mixed ration

ABSTRACT

Two concentrates (MELK and VEM) with two different carbohydrate compositions were supplemented during milking in an Automatic Milking System (AMS). The objectives of this study were to estimate the effect of the concentrates on CH_4 emission from dairy cows and to investigate the precision of the CO_2-method when measuring in an AMS for different length of time. Holstein cows ($n=36$) were used with mean body weight of 660 kg (SD=75.13) and average milk production of 31.7 kg (SD=8.98), mixed parity and mixed lactation. Cows were allocated in two groups ($n=18$). After an adaptation period (period 1), each group received either 100% MELK (More Energy Lactating Cows; a newly introduced feeding system) or 100% VEM (Feed Value System for milk production) during periods 2 and 3. Besides, both groups were fed the same Total Mixed Ration (TMR) *ad libitum* in the stable. Air samples in the AMS from a point near the cows head were analysed every 20 s using the Gasmet equipment based on Fourier Transform Infrared (FTIR) Spectroscopy Technique. The equipment ran continuously for 15 days over the three measurement periods (5 days × 3 periods) with a 14 days waiting time in between the periods. Individual records of the CH_4 and CO_2 concentrations in the cows breath was calculated after subtracting the CH_4 and CO_2 concentrations in the stable air from the measured concentrations. The CH_4:CO_2 ratio was then multiplied with the calculated total CO_2 production by the individual cows to get the quantitative CH_4 production. Milk production and total dry matter intake (DMI, kg/day) were very similar in the two groups. The supplemented concentrate was allocated according to the individual milk yield and the intake ranged from 1.60 to 7.30 kg/day in MELK cows and from 2.06 to 7.20 kg/day in VEM cows. No significant difference was found for CH_4 production in MELK and VEM groups over the three periods. A linear positive relation between the CH_4 (g/day) and energy corrected milk (ECM, kg/day) production and the feed intake (DMI, kg/day) was observed for the entire period. The calculated CO_2 and CH_4 production were very similar in the two groups throughout the entire measurement period. The analysis of the precision of the CO_2-method, using a 95% significance level, indicated that showing a difference of 9 or 5% in methane production requires a measuring period of 5 or 15 days, respectively, when using 18 cows per group. The study shows no effect of a limited change in supplementation of starch and sugar on CH_4 production through feeding concentrates MELK or VEM in the AMS. To obtain an effect of changing the

* Corresponding author. Tel.: +45 35 33 28 68.
** Corresponding author. Tel.: +45 35 33 30 92.
E-mail addresses: naha@sund.ku.dk (M.N. Haque), jom@sund.ku.dk (J. Madsen).

http://dx.doi.org/10.1016/j.livsci.2014.03.004

carbohydrate composition of the diet on the CH_4 production, it is likely that a larger change in the diet is necessary. This can only efficiently be done by changing the TMR part of the diet.

1. Introduction

Methane emissions by the ruminant animals are not only an environmental hazard but represent also a loss of energy from the animal. Globally, 287 Mt of CH_4 are annually released from anthropogenic sources, about 50% of which is from agriculture, and the largest biogenic source of CH_4 is enteric fermentation from ruminant livestock (EPA, 2008). Methane is a natural end product of rumen fermentation. It arises as a result of anaerobic digestion of feed and the removal of hydrogen from the rumen by methanogens. Methane emission from ruminant depends on the diet composition and quantity of feed consumed (Johnson and Johnson, 1995). In dairy cows, the CH_4 energy loss (% of gross energy intake) is about 5.3–6.1% (Benchaar et al., 2013; Hassanat et al., 2013). Efficient dairy production is characterized by high milk production per cow and aims at efficient conversion of feed energy and nutrients to human-edible food, such as milk and meat. This includes efforts to reduce the loss of energy by CH_4 release. Recently, several authors have reviewed a number of strategies to mitigate enteric CH_4 production such as the use of nutritional strategies and genetic modifications in order to change the rumen microbial biodiversity (Martin et al., 2008; Beauchemin et al., 2009; Gerber et al., 2013). At the time being, it seems that the most promising approach for reducing methane emissions from ruminants is by improving productivity and efficiency through better nutritional management (Steinfeld et al., 2006; Gerber et al., 2013). Among nutritional strategies, high concentrate and lipid supplementation is considered most effective in lowering CH_4 production per unit of energy intake (Johannes et al., 2011). The biological mechanism is to shift rumen fermentation towards propionogenesis, whereas fibrous diets result in a preferential production of acetate, butyrate, and CH_4. Feeding of high yielding dairy cows aiming at maximizing milk production often results in high-starch diet. Rapidly degradable starch supplementation leads to a drop in acetate-to-propionate ratio with an ultimate result of reduced methane (Plaizier et al., 2008). Several studies have investigated the effect of different sources of carbohydrate on methane emission (Hristov et al., 2013), but few have examined the possible effect of changing the composition of concentrate allocated to cows in the Automatic Milking System (AMS) where only limited amount of concentrate can be fed. It is hypothesized that feeding starch rich concentrate in the AMS would be effective to reduce methane. A new feed planning system for dairy cows (MELK) has been developed in The Netherlands to substitute the old system (VEM). The new system should favour propionate production, thereby reducing the methane production in the rumen. In addition, there are now a number of methods in focus to estimate the CH_4 production from ruminants e.g. traditional respiration chamber method (Blaxter and Clappert, 1965) and SF6 tracer technique (Johnson et al., 1994). These methods have different pros and cons. Some methods, for instance respiration chamber, can measure with high accuracy but only on few animals and not in their natural environment. Other methods as the CO_2-method newly developed by Madsen et al. (2010) can measure on many animals and give the opportunity to evaluate the variation between animals and differences between diets in practice with a reasonable precision. The CO_2-method is a pertinent technique for measuring on many animals and evaluating differences between feeds. Therefore, the objectives of this study are (i) to investigate the effect of changing the starch, sugar and fibre content on methane emission and milk production using two concentrates MELK and VEM fed in an AMS and (ii) to investigate the precision of the CO_2-method when measuring in an AMS for different lengths of time.

2. Materials and methods

2.1. Experimental design, animals and housing

Holstein-Friesian dairy cows ($n=36$) with an average body weight of 660 (SD=75.13) kg and an average milk yield of 31.7 (SD=8.98) kg/day were selected from a private dairy farm (Dalfsen, The Netherlands) for this study. The cows were initially selected as 18 pairs based on age, parity, daily milk production, body condition and average methane excretion per day. Cows from each pair were randomly assigned into two groups. During a first period, each group received 50% of each concentrate. From day 18th, one group was fed MELK concentrate; the other was fed VEM concentrate. Animals were housed in a closed housing system fitted with one AMS.

2.2. Diets, experimental period and feeding

Both groups were fed the same Total Mixed Ration (TMR) *ad libitum* in the stable and two different concentrate mixtures in AMS named MELK and VEM (Tables 1 and 2). MELK stands for “More Energy Lactating Cows” and is a new feeding system for high yielding dairy cow in the Netherlands. VEM is the “Feed Value System”, considered to be a traditional feeding system which has been used in the Netherlands in the last few decades. TMR was supplied to all cows in the stable two times a day. The concentrates MELK and VEM were automatically supplied during the milking in AMS. The amount of fed concentrates was based on the daily milk yield of individual cows controlled by “The dynamic feeding system” developed by Agrovision©. The experiment was divided into three periods, each of 5 days duration, with 14 days waiting time in

Table 1
Ingredients of Total Mixed Ration (TMR) and the concentrates MELK and VEM.

TMR (% of dry matter)		Concentrates (% of DM)		
Ingredients		Ingredients	MELK	VEM
Grass silage	43.02	Sugar beet pulp	–	31.4
Maize silage	39.85	Palm kernel expeller	–	17.4
Grass seed hay	2.80	Citrus molasses	10.0	10.0
Brewery grain	7.30	Rapeseed expeller	–	9.1
Unimix 922[a]	6.64	Soya hulls	14.8	8.1
Univit Mobiel[b]	0.26	Wheat	6.3	5.7
Calcium carbonate	0.13	Rapeseed meal	16.1	5.1
		Cane molasses	10.0	5.0
		Rapeseed meal	9.0	3.5
		Soya meal	–	2.6
		Premix	2.3	1.6
		Urea	0.3	0.3
		Vegetable oil	0.2	0.2
		Maize	31.1	–

[a] Rapeseed meal and soya meal (1/2% each).
[b] Minerals and vitamins mix.

between each period. During period 1, all cows were supplied both of the concentrates MELK and VEM (50% of each), whereas in periods 2 and 3, 100% MELK or VEM were allocated separately according to the groups.

2.3. Gas measurement

Methane and CO_2 from the cows were analysed using a continuous gas analyser Gasmet DX-4030 (Gasmet™, 2010) based on Fourier Transformed Infrared Radiation. Three days prior to each measurement period, Gasmet was installed to the Delaval AMS to ensure the correctness of measurements. The inlet filter of the Gasmet was fitted on the feeding pen of AMS in order to get concentrated breath sample from cows. The breath sample passes through the filter and thereafter through Gasmet analyser to determine the concentrations of CH_4 and CO_2. The measurements were performed every 20 s over 24 h for the entire 5 days experimental periods. The methane production for the individual cows was based on the methane–carbon dioxide ratio (CH_4:CO_2) when the specific cow was in the AMS. Each cow was visiting the AMS at least two times a day (2.6 times in average), with an average milking time of 6 min. The individual methane production was calculated based on 294 ± 106, 236 ± 74 and 266 ± 88 (mean ± SD) observations per cow during period 1, 2 and 3, respectively. Before the first measurement in each periods, Gasmet was calibrated with standard gas to check the accuracy of the measurement. During period 1, Gasmet was stopped for 10 min each day to get the stable concentration of CH_4 and CO_2. This concentration of CH_4 and CO_2 was subtracted from the measured concentration to get the real breath concentration of CH_4 and CO_2. Remote monitoring of the measurements was performed via internet using TeamViewer (TeamViewer©, 2013).

2.4. Sampling and analysis of feed samples

One sample of the TMR and of the concentrates was taken during each of the measurement periods. Immediately after collection, the samples were stored in a freezer. Before laboratory analysis, the three fractions of the same sample were mixed together to make a composite sample. All of the TMR samples were dried at 65 °C and the concentrates at 103 °C to determine the dry matter percentage. Crude fibre was determined according to EU (2009b) and crude ash at 550 °C according to EU (2009a). Neutral detergent fibre (NDF) was determined following ISO-16472 (2006) where heat stable amylase and ash correction were considered and EFOS through FO-19 (2005). Acid detergent fibre and acid detergent lignin was determined according to ISO-13906 (2008). Crude protein and rate of degradation of protein

Table 2
Chemical composition and nutritive values of TMR the concentrates MELK and VEM.

Chemical composition	TMR (% of dry matter)	Concentrates (% of dry matter)	
		MELK	VEM
Dry matter (% of fresh feed)	47.3	86.3	85.8
Crude protein	12.1	17	17.1
Crude fat	2.8	3.7	3.9
Crude fibre	25.3	11.2	14.4
Ash	6.2	6.5	6.9
Sugar	5.8	7.6	11.9
Starch	11.48	29.66	6.27
ADF	26	16	22
NDF	45.1	20.7	30.8
Lignine	2.4	3.2	3.9
Calcium	0.48	0.84	0.81
Phosphorus	0.31	0.39	0.37
Nutritive values			
EFOS (% of organic matter)	71.4	94	91.7
Buffer solubility (% of crude protein)	51.2	21.0	25.5
Digestible energy[a], MJ/kg DM	13.42	16.5	16.2
Metabolizable energy[b], MJ/kg DM	10.74	13.2	13.0
Scandinavian Feed Units[c], SFU/kg DM	0.87	1.22	1.19

ADF = acid detergent fibre.
NDF = Neutral detergent fibre.
DM = dry matter.
EFOS = enzyme solubility of organic matter.

[a] Digestible energy = 24.237 × digestible crude protein (kg/kg DM) + 34.116 × digestible crude fat (kg/kg DM) + 17.300 × digestible carbohydrate (kg/kg DM) − 0.766 × sugar (kg/kg DM).
where

Digestible organic matter for TMR (%) = 0.204 + 0.727 EFOS.
Digestible organic matter for concentrate (%) = 5.38 + 0.867 EFOS. (Weisbjerg et al., 2007).
Digestible crude protein (kg/kg DM) = (0.93 × % crude protein in DM − 3)/100.
Digestible crude fat (kg/kg DM) = (0.96 × % crude fat in DM − 1)/100.
Digestible carbohydrate (kg/kg DM)) = (% digestibility of organic matter/100) × (100 − % crude ash in DM)/100 − digestible crude protein − digestible crude fat.

[b] Metabolizable energy = Digestible energy × 0.80.
[c] Scandinavian Feed Units = −0.369 + 0.0989 × Digestible energy − 0.347crude fibre (kg/kg DM).

through buffer solubility test was determined according to Licitra et al. (1996). Crude fat was determined following ISO-11085 (2008) using petroleum ether. Enzymatic method (ISO-15914, 2004) was followed to determine the amount of starch whereas the titration method (EU, 2009c) was followed to determine the amount of sugar.

2.5. Calculations

The data for air composition was matched with the cow identification numbers and data for entrance and exit times of the individual cows into the AMS by using the time recorded in a computer connected to the AMS. All calculations regarding CH_4 and CO_2 emissions from cows were done according to the CO_2-method (Madsen et al., 2010). The stable concentrations of CO_2 (605 ± 88.3 ppm) and CH_4 (26 ± 10.3 ppm) (mean ± SD) obtained from period 1 were subtracted from the exhaled concentration of the cows to get the corrected breath concentration of each sample. After correction, all values of corrected CO_2 below 400 ppm were removed in order to avoid the influence of samples containing a very low concentration of breath. The ratio between CH_4 and CO_2 (CH_4:CO_2) was thereafter determined. This ratio represents an index of feed gross energy loss in CH_4 as well as a factor for quantifying CH_4 from the animals (Madsen and Bertelsen, 2012).

The body weight (BW) (kg) of the animals was determined according to Remmelink et al. (2011), as shown in Eq. (1). The dry matter intake (DMI) kg/day of concentrate was set to the amount allocated on individual and daily basis. The average TMR intake for the cows in the two groups was set as the herd average. The individual TMR intake of the cows was calculated following Eq. (2) described by Kristensen and Ingvartsen (2003), where the intake is corrected according to the amount of concentrate allocated and the parity of the individual cows. The individual total dry matter intake (TDMI) was calculated by adding the individually allocated concentrate dry matter intake (CDMI) to individually calculated TMR dry matter intake (TMRDMI). The heat production (HP) watt of the cows was calculated following Eq. (3), described by CIGR (2002). The excretion of CO_2 (L/day) was calculated according to Pedersen et al. (2008), as shown in Eq. (4). The amount of methane (g/day) was calculated as described by Madsen et al. (2010) using Eq. (5). Energy corrected milk (ECM) (kg) was calculated following the Eq. (6) described by Sjaunja et al. (1991).

$$BW(kg) = 0.000275 \times Breast\ size\ in\ \mathrm{cm}^{2.76} \tag{1}$$

$$TMRDMI\left(\frac{\mathrm{kg}}{\mathrm{day}}\right) = a + 0.5(b - c) + d \tag{2}$$

$$HP(\mathrm{watt}) = 5.6 \times BW^{0.75} + \{(Y \times 22) + (1.6 \times 10^{-5} \times P^3)\} \tag{3}$$

$$CO_2(L/\mathrm{day}) = HPU \times 180 \times 24 \tag{4}$$

$$CH_4(\mathrm{g/day}) = CO_2 \times \frac{CH_4}{CO_2} \times 0.714 \tag{5}$$

$$ECM\ (\mathrm{kg}) = Y \times (0.383 \times milk\,fat + 0.242 \times milk\ protein + 0.7832)/3.14 \tag{6}$$

where

a is the measured average TMR intake;
b is the measured average concentrate intake;
c is the allocated concentrate intake of the individual cows during the experimental periods;
d is the correction factor for the lactation number: $d = -1.61$ was considered for first lactation and $d = 0.39$ for the second and subsequent lactations;
HP is heat production from the animals;
$BW^{0.75}$ is metabolic body weight of the animals;
Y is Milk yield of cow kg/day
P is days of pregnancy
HPU = Heat producing unit ($HP/1000$);
180 = L of CO_2/HPU/h;
ECM = Energy corrected milk.

2.6. Statistical analyses

Statistical analyses were performed using the software R (R Development Core Team, 2013). The data were fitted using mixed models using the lme function from the package nlme (Bates and Sarkar, 2009).

The analyses focused on making inference about the effect of the concentrates (MELK and VEM) and about the length of the treatment for changes in levels of CH_4 (g/day), CH_4:CO_2, CH_4 (g/kg DMI) and CH_4 (g/kg ECM). Therefore all periods 1, 2 and 3 were included in the analysis. For all of the response variables an average data per cow and per day were used. Group, period of measurement, the interaction group × period, BW, DMI, ECM and lactation numbers were included as fixed effects in the primary model. Both cow number and day of measurement were included as random effects. Different serial correlation structures were tested for the effect of day. The final model was confirmed by stepwise removing of the non-significant variables. Model validation was performed using analysis of variance (ANOVA) on the Akaike Information Criterion. Model residuals were checked for normality and homoscedasticity by visual inspection, qqplots and Bartlett test. The final model was:

$$y_{ijk} = \mu + \alpha_i + \beta_j + X\gamma_{ijk} + Y\theta_{ijk} + C_k + \varepsilon_{ijk}, \tag{7}$$

where y_{ijk} is the response variable $y = \{CH_4$ g/day, CH_4:CO_2, CH_4 g/kg DMI, CH_4 g/kg ECM$\}$ of i group, for period j and cow k and μ is the overall mean. The fixed effects are the group α_i with $i = \{$MELK, VEM$\}$, the period β_j with $j = \{$period 1, period 2, period 3$\}$, the ECM (kg) for cow k, $X\gamma_{ijk}$, and the BW for cow k, $Y\theta_{ijk}$; C_k is the random effect of cow k and ε_{jkl} is the residual errors. Even though only ECM was significant, BW was included since it has a direct influence on CO_2 production. Group and period were also included since both of them are of interest for this study. The least square means (LSM) were extracted from the model by using the package lsmeans as described by Russell (2013). Multiple comparison was done using Tukey's pairwise

Table 3
Average body weight, milk production (corrected and uncorrected), TMR and concentrate intake of cows per day; n is the number of observations.

Parameters	MELK [mean (SE)]	VEM [mean (SE)]	*n*
BW (kg)	647 (19.2)	674 (15.9)	18
Milk production (kg/day)	31.8 (0.56)	31.6 (0.54)	270
ECM (kg/day)	33.1 (0.48)	33.7 (0.51)	270
TMRDMI (kg/day)	18.7 (0.06)	18.7 (0.06)	270
CDMI (kg/day)	4.5 (0.12)	4.8 (0.12)	270
TDMI (kg/day)	23.2 (0.14)	23.5 (0.14)	270

SE = standard error.
BW = body weight.
ECM = energy-corrected milk.
TMRDMI = total mixed ration dry matter intake.
CDMI = concentrate dry matter intake.
TDMI = total dry matter intake.

comparisons using the function glht from the multcomp package (Hothorn et al., 2008). One way ANOVA were carried out to get the model *P* values. Finally, using the information from this study, precision based power calculation was performed in order to estimate the minimum mean difference that indicates a significant effect between groups, according to the number of observations (Pandis et al., 2011).

3. Results

3.1. Dry matter intake and milk production

Data for BW, milk production, DMI for TMR and concentrates are shown in Table 3. Total DMI were 23.2 (SE = 0.08) and 23.5 (SE = 0.08) kg/day in MELK and VEM respectively, with individual cows values ranging from 21.5 to 25.6 kg/day. There was a large variation among the cows in milk production and consequently concentrate dry matter intake, as the amount of concentrate was supplied according to individual milk production.

3.2. CH_4:CO_2 ratio and methane emission

Measurements of air composition were performed in the AMS every 20 s throughout the entire experimental period. The frequent air analysis was done in order to get ample data for the best possible estimation of CH_4 production as the individual observations of breath sample analysis show large variation from each other. Fig. 1 illustrates the variation in concentration of breath in the analysed air sample by showing the concentration of CO_2 in 52 measurements of air for a single cow during three visits. Likewise the variation in the corrected CH_4:CO_2 ratio for the same 52 observations is shown in Fig. 2. There is still a variation between samples and this can be ascribed to the different concentrations of CH_4 in the breath. All exhaled air from the cows contains CH_4 and the high values of more than 0.2 indicate that the CH_4:CO_2 ratio can get close to the ratio in the rumen.

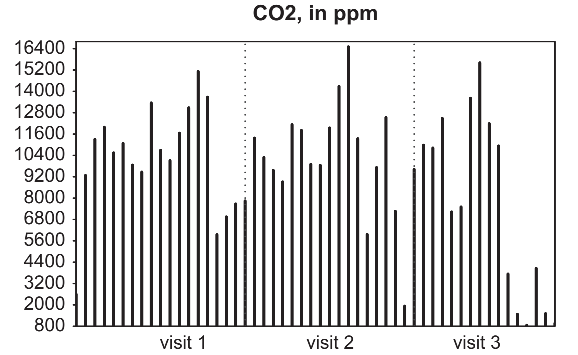

Fig. 1. Individual observations of CO_2 (ppm) concentration in analysed air from three visits of a cow from group VEM during period 1.

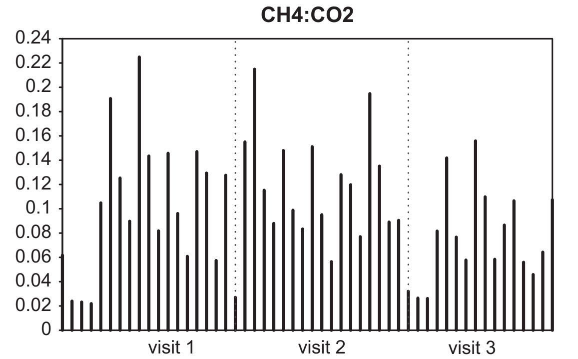

Fig. 2. Individual values of the calculated CH_4:CO_2 ratio corresponding to the measurements from Fig. 1.

Results of the mixed models (Table 4) indicate that there was no significant difference in the effect of supplementation of the two concentrates on the CH_4 production ($P = 0.97$). The difference between the three periods was also non-significant ($P = 0.49$). In addition, about 43% of the random variation in CH_4 (g/day) production is due to the individual cow effect, the rest being due to the variation between measurements. Results for the ratio CH_4:CO_2 also indicate that both the effects of the group ($P = 0.75$) and period ($P = 0.62$) are non-significant. In order to visualize the day to day variation, the arithmetic mean of CH_4:CO_2 and of CH_4 production (g/day) in the two groups and over the three periods are shown in Fig. 3.

The methane production per cow increased with increased ECM as a result of the higher DM intake with increased milk production. The coefficient for ECM ($P < 0.001$) from the mixed model indicates an incremental emission of methane of 6.1 g/kg increase in ECM. As the concentrate is fed according to milk yield, the difference of ingested starch content is smaller for low yielding cows and larger for high yielding cows. Therefore, a different effect on low and high yielding cows could be expected in the current study.

Scatterplots of CH_4 production (g/day) according to ECM is shown in Fig. 4. A simple linear regression using average CH_4 release (g/day) showed very similar slopes in two groups during period 1, where both groups got the

Table 4
Least square means (LSM) of CH_4:CO_2 and CH_4 production g/day, g/kg DMI and g/kg ECM.

	CH_4:CO_2 ratio [LSM (SE)]	CH_4 g/day [LSM (SE)]	CH_4 g/kg feed DMI [LSM (SE)]	CH_4 g/kg ECM [LSM (SE)]
MELK				
Period 1	0.0993 (0.00435)	444 (19.5)	19.0 (0.98)	13.8 (0.78)
Period 2	0.1001 (0.00436)	449 (19.5)	19.9 (0.98)	14.0 (0.78)
Period 3	0.0997 (0.00436)	450 (19.5)	19.6 (0.98)	14.1 (0.78)
Mean[a]	0.1000 (0.00405)	447 (18.2)	19.6 (0.98)	14.2 (0.70)
VEM				
Period 1	0.0987 (0.00436)	436 (19.6)	18.1 (0.84)	13.5 (0.78)
Period 2	0.0995 (0.00436)	442 (19.5)	19.0 (0.98)	13.7 (0.78)
Period 3	0.0991 (0.00436)	442 (19.5)	18.7 (0.98)	13.8 (0.78)
Mean[a]	0.0993 (0.00405)	438 (18.2)	18.7 (0.84)	13.9 (0.70)

SE = standard error.
ECM = energy corrected milk.
DMI = dry matter intake.

[a] Mean values considering both periods 2 and 3.

same diet consisting of MELK and VEM (50% of each). Based on the linear regression, in periods 2 and 3 when two groups were either MELK or VEM (100%), a tendency is shown between the groups ($P=0.07$ for both subsets of period 2 and 3). The VEM group tended to show a sharper slope than the MELK group. This supports the hypothesized highest effect of a high starch concentration in the concentrate on lowering CH_4 when feeding the highest amount of concentrate.

3.3. *Precision of the CH_4 estimates*

Table 5 reports the results of precision based power calculations for CH_4 production (g/day) using the results SD = 74 based on the mean values per cow per day from the present experimental conditions. The results indicate that in order to get a significant difference at 5% level, the minimum mean difference of CH_4 (g/day) between the groups should be at least $\pm$ 40, 28 and 23 equivalent to a 9, 6 and 5% for 5, 10 and 15 days of measurement, respectively, with 18 cows per group.

4. Discussion

4.1. *Breath sample measurement*

The air samples analysed are influenced by the concentration of breath in the air samples as the position of the nose of the cows in relation to the inlet filter varies. Most samples have a CO_2 concentration of between 5000 and 10,000 ppm which shows that samples contain between 10 and 30% of breath considering that the average concentration of CO_2 in breath typically ranges between 30,000 and 50,000 ppm (Elliott-Martin et al., 1997; Smith et al., 2009). When calculating the corrected CH_4:CO_2 ratio after subtracting the concentration of CH_4 and CO_2 in the surrounding air, the variation due to the position of the nose in relation to the inlet filter is removed. Part of the variation in the CH_4:CO_2 ratio is also caused by the proportion of the ruminal fermentation gases and gases from normal breathing. This variation requires several measurements to obtain a reliable mean value. A recent large scale study for breeding purposes describes that more than 3 days measurement is expensive and impractical for getting better estimation of the CH_4:CO_2 ratio (Lassen et al., 2012). Furthermore, Madsen et al. (2010) mentioned that about 2–3% of breath in the analysed air sample is sufficient to get a precise estimation of CH_4 production. The latter also indicate that it is sufficient to get a relative diluted breath to get a reliable determination of the CH_4:CO_2 ratio.

4.2. *Methane production*

As described by Hindrichsen et al. (2004), the mode of fermentation of starch and sugar and their end products indicate that there should be a lower CH_4 production in the MELK group. In the current study, there was a tendency observed that the cows receiving the highest amount of MELK concentrate (high starch) produced less CH_4 (g/day). Nevertheless, no significant differences were found in CH_4 output between the groups. The reason for the absence of reduced CH_4 production in the MELK group is probably due to the very limited change in the total carbohydrate composition of the diet. As seen in Table 6 the starch content that was expected to be lowered the CH_4 output was four times as high in the MELK concentrate as in the VEM concentrate. When calculated on the total diet basis (concentrate + TMR) the total starch content increases only with 25%. The starch proportion of the total potential digestible carbohydrates (sugar, starch and NDF) is 21.7% in VEM to 27.5% in MELK. It can therefore be assumed that only 5.8% increase in the starch content of the total diet has not been enough to change the CH_4 emission. Similar effects have been presented by Aguerre et al. (2011) where dietary starch content was reduced by increasing fibre concentration in the diet of dairy cows. Mc Geough et al. (2010) reported a decreased CH_4 (g/kg DMI) emission in beef cows with increased amount of starch feeding through whole-crop wheat silages. In the same line, Hassanat et al. (2013) reported a reduced methane emission in dairy cows by supplementing 30% starch

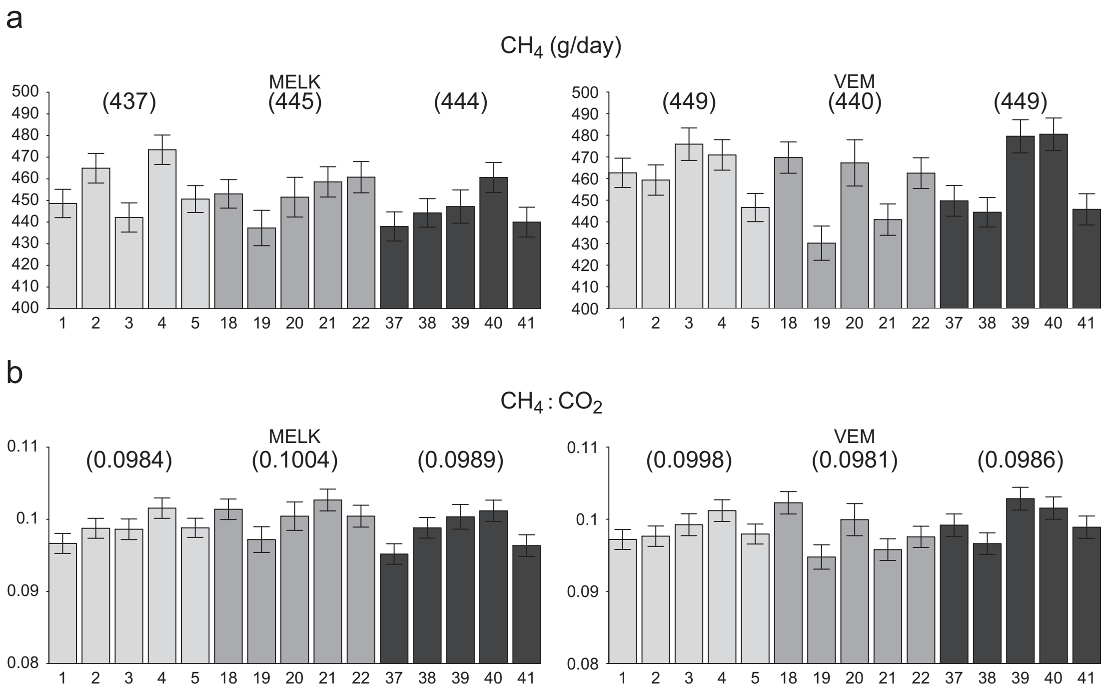

Fig. 3. Daily averages (± SE) of CH_4:CO_2 ratio and CH_4 production (g/day) for the MELK and VEM groups for the 15 days measurement period. Each period has a different shade of grey and average per period is indicated in brackets. The horizontal axis indicates the experimental day.

through 100% corn silage, whereas no effect on CH_4 has found by increasing starch from 17–27% starch through supplementation of 0–50% corn silage. As mentioned earlier, in the current study only 25% increase in starch (on total ration basis) resulted in no effect on CH_4 (g/day) reduction, which is in accordance with Hassanat et al. (2013). The latter suggested that the methane reduction effect of starch is linked with acidic ruminal environment due to low pH (< 6.0) and with the shift of the volatile fatty acid pattern toward proportionally more propionate and less acetate and butyrate. Furthermore, Fahey and Berger (1988) pointed out starch as the most propionate producer in rumen fermentation than any other carbohydrates. In the present study, the supplemented amount of starch through concentrate in AMS was certainly too low to show a response on changing rumen environment with increase propionate proportion and consequently CH_4 reduction. Besides, supplying diet containing high quantities of starch via grain or cereal forages has been proposed as a mean of methane reduction (Beauchemin et al., 2008). The current study made a similar effort to reduce methane by increasing the amount of starch. However, it appears that an inapt way was chosen to supplement starch through concentrate in AMS. Feeding starch through TMR could have been more effective to visualize the methane reduction effect.

4.3. *Precision of the CH_4 estimates*

The CO_2-method used in the experiment is relatively newly developed (Madsen et al., 2010). It offers opportunities to get measurements from many animals within
a short time, which is an advantage in many situations. A large number of animals is typically required for breeding experiments (Lassen et al., 2012). Furthermore, increasing the number of individuals can improve the precision of CH_4 measurement for feeding experiments.

When comparing the effect of different diets on groups of cows, it is of uttermost importance that the precision of the estimates are high whereas the level (or accuracy) is of less importance to validate the effect of diets or treatments.

Danielsson et al. (2012) showed a large individual variation between cows using the SF_6 method. The variation of CH_4 ranges from 12.3 to 21.8 for one diet, and from 11.8 to 25.7 (g/kg DMI) for another diet, for averages based on a five days measurement period. To reduce this variation would require increasing the number of animals or days of measurement. In this study, the individual cow variation of CH_4 (g/kg DMI), based on the 15 days experimental period ranged from 17.0 to 23.0 in the MELK group and from 14.0 to 21.6 for VEM group. The individual variation of CH_4 production was highlighted as most important by Grainger et al. (2007) and has also been seen in own experiment (Haque et. al., 2014, unpublished data).

The shown variation in the CH_4:CO_2 ratio and the estimated daily CH_4 output are assumed to be related to the time of the day the cows visit the AMS. Moreover, the time of the day when they have eaten TMR may influence the actual CH_4 production.

In this study, the resulted least square means difference of CH_4 (g/day) between groups (Table 4) was about 8,

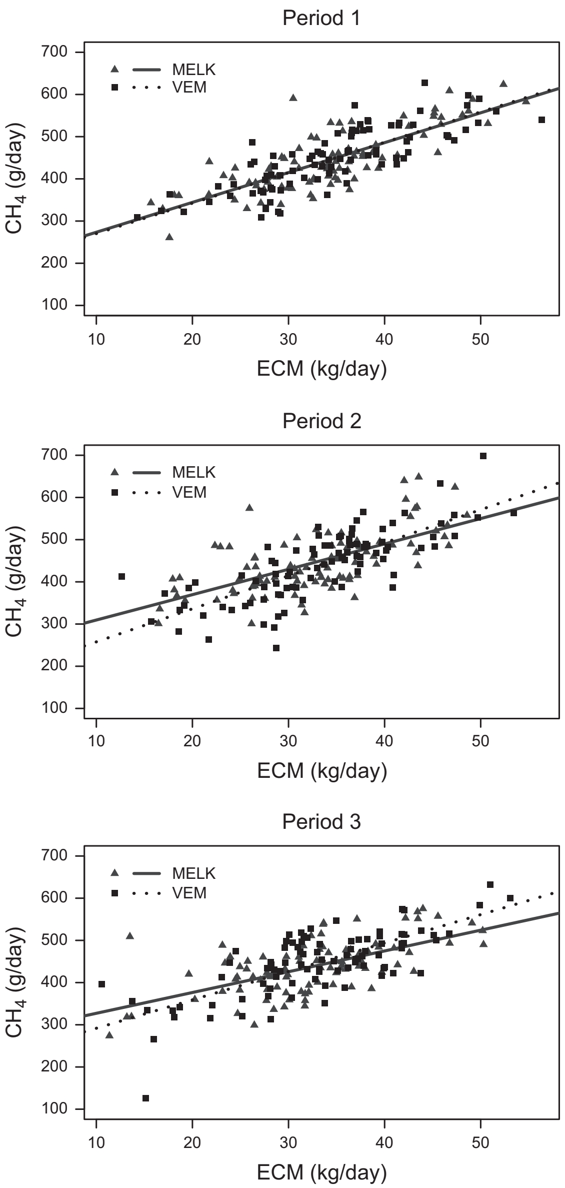

Fig. 4. Linear regression of CH_4 production (g/day) for cows of MELK and VEM groups in the three periods of measurement.

Table 5
Expected mean difference in methane production (g/day) according to the number of observations (cows × days) per group in order to get a significant difference between the groups at a 0.05 level of significance and a power of 95%.

Number of cows	Days of measurement	Expected mean difference[a]	Expected mean difference[b] (%)
18	1	89	20
18	5	40	9
18	10	28	6
18	15	23	5

[a] Calculated from $d=\sqrt{f(\alpha,\beta)\{2\times SD^2\}/n}$. Where α=*significance level*, β=*Power*, $f(\alpha,\beta)=13.0$ at 5% level of significance for a power of 95%. SD=standard deviation of the response (74) and n=number of observations per group.

[b] Calculated considering a mean values 442, for periods 2 and 3 (see Table 4).

7 and 8 for period 1, 2 and 3 respectively, and 9 when considering both period 2 and 3 (10 days in total). At the light of the findings of precision-based power calculation, the results show no significant difference was obtained between groups. In order to improve the precision of CH_4 quantification, more individuals or measurements for a longer period of time is needed.

4.4. Accuracy of the methane production

For an experiment as the present, another aspect is whether the estimates are accurate, i.e. whether they give values of the right magnitude. The accuracy is also influenced by the accuracy of the calculated CO_2 production. The formula used is based on the work of an international commission of agriculture (CIGR, 2002) and of Pedersen et al. (2008), and is considered reasonably accurate and the best available. Comparing the results of this experiment with recently published studies using the SF_6 (O'Neill et al., 2011; Danielsson et al., 2012) and chambers methods (Aguerre et al., 2011; van Zijderveld et al., 2011) indicate comparable magnitude and associated precision of CH_4 emission (g/kg DMI). In this study, the emissions range from 18.7–19.6 (MELK–VEM), whereas the other studies report values ranging from 16.9 (Danielsson et al., 2012) to 25.9 (Aguerre et al., 2011) g/kg DMI. The corresponding SEM for CH_4 (g/kg DMI) reported for the chamber experiments are 0.65 (van Zijderveld et al., 2011) and 1.21 (Aguerre et al., 2011); for the SF6, the values are 0.57 (O'Neill et al., 2011) and 2.9 (Danielsson et al., 2012; only SED was reported). The value reported in the present study (0.84 for 10 days measurement) indicates that the CO_2-method is as precise as the other methods and produces results of the same magnitude. In O'Neill et al. (2011), for which the SEM is the lowest, it should be noted that the number of individuals per group ($n=24$; 10 days measurements) was larger than in the present study ($n=18$; 10 days measurements).

As high yielding cows have a lower emission of CH_4 per kg milk, lowering the marginal emission may also be an objective in itself. Tamminga et al. (2007) made a prediction of the expected CH_4 production per kg milk at different levels of milk production. At the milk production level corresponding to the cows of this study (33.5 kg/day) the predicted CH_4 production was 12.8 g/kg ECM. In this respect, the CH_4 production reported in this study, ranging from 13.9 to 14.2 g/kg ECM (Table 4) can be considered estimated with an acceptable accuracy.

5. Conclusions

This study showed no effect of changing the composition of concentrate fed in AMS to higher starch content and less fibre and sugar on the methane output. The absence of hypothesized reduction in the CH_4 (g/day) release is most likely due to the small proportion of dry matter consumed from the allocated concentrate in the AMS,

Table 6
Average carbohydrate intake of the diets, kg DM/cow/day.

	MELK			VEM			Change (%) in nutrient, VEM to MELK	
	Total	TMR	Concentrate	Total	TMR	Concentrate	Total	Concentrate
Sugar	2.8	2.4	0.4	3.1	2.4	0.7	−10	−43
Starch	6.4	4.7	1.7	5.1	4.7	0.4	+25	+325
NDF	19.8	18.6	1.2	20.5	18.6	1.9	−3	−37
Total DM	23.2			23.5				
% Starch in DM	27.5			21.7			+5.8	

TMR = total mixed ration.
NDF = neutral detergent fibre.

which is scanty in relation to the dry matter intake from the TMR. To obtain an effect on CH_4 yield by dietary manipulation with carbohydrate composition, it is recommended to change the composition of the TMR part of the diet, as this can result in a greater change in the composition of the total carbohydrate intake. The results from the used CO_2-method illustrate that higher precision can be obtained by either having more cows in the experiment or by measuring for a longer period.

Conflict of interest statement

The authors report that there is no conflict of interest relevant to this publication.

Acknowledgements

The study was supported by a stipend from University of Copenhagen, Denmark and a research grant from the Dutch Ministry of Finance and Innovation through the company Feed Innovation Services, the Netherlands.

References

Aguerre, M.J., Wattiaux, M.A., Powell, J.M., Broderick, G.A., Arndt, C., 2011. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. J. Dairy Sci. 94, 3081–3093.
Bates, D.M., Sarkar, D., 2009. lme4: Linear Mixed-effects Models Using S4 Classes, R package version 0.99875-6. . ⟨http://cran.r-project.org/web/packages/lme4/index.html⟩ (accessed 17.04.13).
Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Exp. Agric. 48, 21–27.
Beauchemin, K.A., McAllister, T.A., McGinn, S.M., 2009. Dietary mitigation of enteric methane from cattle. CAB Rev.: Perspect. Agric., Vet. Sci., Nutr. Nat. Resour. 4, 1–18.
Benchaar, C., Hassanat, F., Gervais, R., Chouinard, P.Y., 2013. Effects of increasing amounts of corn dried distillers grains with solubles in dairy cow diets on methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 2413–2427.
Blaxter, K.L., Clappert, J.L., 1965. Prediction of amount of methane produced by ruminants. Br. J. Nutr. 19, 511–522.
CIGR, 2002. Climatization of animal houses—heat and moisture production at animal and house level. In: Pedersen, S., Sällvik, K. (Eds.), 4Th Report of CIGR Working Group. Horsens, Denmark. Research Centre Bygholm, Danish Institute of Agricultural Sciences, P.O Box 536, DK-8700 Horsens, Denmark.
Danielsson, R., Schnurer, A., Arthurson, V., Bertilsson, J., 2012. Methanogenic population and CH_4 production in swedish dairy cows fed different levels of forage. Appl. Environ. Microbiol. 78, 6172–6179.
Elliott-Martin, R.J., Mottram, T.T., Gardner, J.W., Hobbs, P.J., Bartlett, P.N., 1997. Preliminary investigation of breath sampling as a monitor of health in dairy cattle. J. Agric. Eng. Res. 67, 267–275.
EPA, 2008. Inventory of U.S. Greenhouse Gas Emissions and Sinks. U.S. Environmental Protection Agency, Washington, DC 20460, U.S.A. (1200 Pennsylvania Avenue, N.W).
EU, 2009a. Determination of crude ash. Off. J. Eur. Union Comm. Regul. 152, 50–52.
EU, 2009b. Determination of crude fibre. Off. J. Eur. Union Comm. Regul. 152, 40–42.
EU, 2009c. Determination of sugar. Off. J. Eur. Union Comm. Regul. 152, 45–52.
Fahey, G.C., Berger, L.L., 1988. Carbohydrate nutrition of ruminants. In: Church, D.C. (Ed.), The Ruminant Animal: Digestive Physiology and Nutrition, Prentice Hall Inc., Englewood Cliffs, NJ.
FO-19, 2005. Changing the Method of Analysis to the Provisions of EFOS Cattle. Ministry for food agriculture and fisheries, Denmark.
Gasmet™, 2010. Gasmet Technologies Oy Pulttitie 8A, FI-00880 Helsinki, Finland (accessed 17.04.13).
Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A., Tempio, G., 2013. Tackling Climate Change Through Livestock—A Global Assessment of Emissions and Mitigation Opportunities. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.
Grainger, C., Clarke, T., McGinn, S.M., Auldist, M.J., Beauchemin, K.A., Hannah, M.C., Waghorn, G.C., Clark, H., Eckard, R.J., 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF6) tracer and chamber techniques. J. Dairy Sci. 90, 2755–2766.
Hassanat, F., Gervais, R., Julien, C., Massé, D.I., 2013. Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 4553–4567.
Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Soliva, C.R., Knudsen, K.E.B., Madsen, J., Kreuzer, M., 2004. Effects of feed carbohydrates with contrasting properties on rumen fermentation and methane release in vitro. Can. J. Anim. Sci. 84, 265–276.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical J. 50, 346–363.
Hristov, A.N., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J., Oosting, S., 2013. In: Gerber, J. Pierre, Henderson, Benjamin, Makkar, H.P.S (Eds.), Mitigation of Greenhouse gas Emissions in Livestock Production—A Review of Technical Options for Non-CO_2 Emissions, FAO Animal Production and Health, FAO, Rome, Italy.
ISO-11085, 2008. Animal Feeding Stuffs—Determination of Crude Fat and Total Fat Content by the Randall Extraction Method. International Organization for Standardization, Geneva, Switzerland (ISO-11085:2008).
ISO-13906, 2008. Animal Feeding Stuffs—Determination of Acid Detergent Fiber and Acid Detergent Lignin Contents. International Organization for Standardization, Geneva, Switzerland (ISO 13906:2008).
ISO-15914, 2004. Animal Feeding Stuffs—Enzymatic Determination of Total Starch Content. International Organization for Standardization, Geneva, Switzerland (ISO 15914:2004).

ISO-16472, 2006. Animal Feeding Stuffs—Determination of Amylase-treated Neutral Detergent Fibre Content. International Organization for Standardization, Geneva, Switzerland (ISO 16472:2006).

Johannes, M., Louise, P.L.A., Hellwing, F., Weisbjerg, M.R., 2011. Hvad betyder fodring for metanemission?. In: Kristensen, T., Lund, P. (Eds.), Kvæg og Klima. Videnskabelig Rapport no. 1. Aarhus Universitet, DCA—Nationalt Center for Fødevarer og Jordbrug, Denmark.

Johnson, K., Huyler, M., Westberg, H., Lamb, B., Zimmerman, P., 1994. Measurement of methane emissions from ruminant livestock using a sulfur hexafluoride tracer technique. Environ. Sci. Technol. 28, 359–362.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483–2492.

Kristensen, V.F., Ingvartsen, K.L., 2003. Forudsigelse af foderoptagelsen hos malkekøer og ungdyr. In: Hvelplund, T., Nørgaard, P. (Eds.), Kvægets Ernæring og Fysiologi. Bind 1. Ministeriet for Fødevarer Landbrug og Fiskeri, Næringsstofomsætning og fodervurdering: DJF rapport, Husdyrbrug, Denmark, pp. 511–564.

Lassen, J., Lovendahl, P., Madsen, J., 2012. Accuracy of noninvasive breath methane measurements using Fourier transform infrared methods on individual cows. J. Dairy Sci. 95, 890–898.

Licitra, G., Hernandez, T.M., VanSoest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347–358.

Madsen, J., Bertelsen, M.F., 2012. Methane production by red-necked wallabies (*Macropus rufogriseus*). J. Anim. Sci. 90, 1364–1370.

Madsen, J., Bjerg, B.S., Hvelplund, T., Weisbjerg, M.R., Lund, P., 2010. Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. Livest. Sci. 129, 223–227.

Martin, C., Rouel, J., Jouany, J.P., Doreau, M., Chilliard, Y., 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. J. Anim. Sci. 86, 2642–2650.

Mc Geough, E.J., O'Kiely, P., Hart, K.J., Moloney, A.P., Boland, T.M., Kenny, D.A., 2010. Methane emissions, feed intake, performance, digestibility, and rumen fermentation of finishing beef cattle offered whole-crop wheat silages differing in grain content. J. Anim. Sci. 88, 2703–2716.

O'Neill, B.F., Deighton, M.H., O'Loughlin, B.M., Mulligan, F.J., Boland, T.M., O'Donovan, M., Lewis, E., 2011. Effects of a perennial ryegrass diet or total mixed ration diet offered to spring-calving Holstein-Friesian dairy cows on methane emissions, dry matter intake, and milk production. J. Dairy Sci. 94, 1941–1951.

Pandis, N., Polychronopoulou, A., Eliades, T., 2011. Sample size estimation: an overview with applications to orthodontic clinical trial designs. Am. J. Orthod. Dentofac. 140, 141–146.

Pedersen, S., Blanes-Vidal, V., Joergensen, H., Chwalibog, A., Haeussermann, A., Heetkamp, M.J.W., Aarnink, A.J.A., 2008. Carbon dioxide production in animal houses: a literature review. Agric. Eng. Int.: CIGR Ej. X, 1–19. (Manuscript BC 08 008).

Plaizier, J.C., Krause, D.O., Gozho, G.N., McBride, B.W., 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. Vet. J. 176, 21–31.

R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R version 3.0.0. 〈http://www.R-project.org〉.

Remmelink, G., Blanken, K., Van Middelkoop, J., Ouweltjes, W., Wemmenhove, H., 2011. Handboek Melkveehouderij. Handboek. Wageningen UR Livestock Research. (p. 6-6).

Russell, V.L., 2013. Least-squares means, R-package. 〈http://cran.r-project.org/web/packages/lsmeans/〉 (accessed 14.05.13).

Sjaunja, L.O., Baevre, L., Junkkarinen, L., Pedersen, J., Setala, J., 1991. A Nordic proposal for an energy corrected milk (ECM) formula. In: Gaillon, P., Chabert, Y. (Eds.), Performance Recording of Animals State of the Art 1990. EAAP Publication 50, Centre for Agricultural Publishing and Documentation, Wageningen, pp. 156–157.

Smith, D., Pysanenko, A., Spanel, P., 2009. The quantification of carbon dioxide in humid air and exhaled breath by selected ion flow tube mass spectrometry. Rapid Commun. Mass Spectrom. 23, 1419–1425.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., de Haan, C., 2006. Livestock's Long Shadow: Environmental Issues and Options. Food and Agriculture Organization of the United Nations. FAO, Rome, Italy.

Tamminga, S., Bannink, A., Dijkstra, J., Zom, R., 2007. Feeding Strategies to Reduce Methane Loss in Cattle. Report: Animal Nutrition and Animal Sciences Group, Wageningen UR.

TeamViewer©, 2013. Kuhnbergstr, 16 73037 Göppingen Germany. 〈http://www.teamviewer.com/en/news/〉 (accessed 17.04.13).

van Zijderveld, S.M., Fonken, B., Dijkstra, J., Gerrits, W.J.J., Perdok, H.B., Fokkink, W., Newbold, J.R., 2011. Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. J. Dairy Sci. 94, 1445–1454.

Weisbjerg, M.R., Søegaard, K., Thøgersen, R., Mikkelsen, M., Brunsgaard, G., 2007. Bestemmelse af Fordøjelighed af Organisk stof i Grovfoder ved brug af In vitro-Metoder Baseret på Vomvæske eller Enzymer. DJF Husdyr No. 76. Aarhus Universitet, Det Jordbrugsvidenskabelige Fakultet.